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Diversity of Extended-Spectrum β-Lactamases and Class C β-Lactamases among Cloacal *Escherichia coli* Isolates in Belgian Broiler Farms▽

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A total of 295 ceftiofur-resistant *Escherichia coli* isolates were obtained from 489 cloacal samples collected at five different Belgian broiler farms with the aim to evaluate the diversity of this resistance at the farm level. Strains were examined for resistance against β-lactam antibiotics and other antimicrobial agents by using disk diffusion tests. Three different β-lactam resistance phenotypes suggested the presence of an extended-spectrum β-lactamase (ESBL), a class C β-lactamase, or the combination of an ESBL with a class C β-lactamase. Seventy-six percent of these isolates also showed acquired resistance to other antimicrobial agents. After genotyping by repetitive extragenic palindromic-PCR, 51 unrelated *E. coli* strains were selected for further analyses. Isoelectric focusing and sequencing of the amplicons obtained in PCRs for the detection of genes encoding broad-spectrum β-lactamase enzymes revealed the following ESBLs: TEM-52 (13.2%), TEM-106 (2%), CTX-M-1 (27.4%), CTX-M-2 (7.8%), CTX-M-14 (5.9%), and CTX-M-15 (2%). The only plasmidic AmpC β-lactamase found in this study was the CMY-2 enzyme (49%). Mutations in the promoter and attenuator regions of the chromosomal *ampC* gene were found only in association with bla_{CMY-2} genes and ESBL genes. The combination of an ESBL (CTX-M-1) with a plasmidic AmpC β-lactamase (CMY-2) was found in 7.8% of the isolates. These data show that ceftiofur-resistant *E. coli* strains are often present in cloacal samples of broilers at the farm level in Belgium. The diversity of broad-spectrum β-lactamases among these isolates is high, and they may act as a reservoir of ESBL and *ampC* genes.

Escherichia coli strains are gram-negative bacteria that act mostly as normal commensals in the intestinal tract of animals and humans, while others are important intestinal and extraintestinal pathogens (25). Human- and animal-pathogenic *E. coli* strains are able to cause a spectrum of illnesses ranging from self-limiting gastrointestinal infections to bacteremia. For the treatment of these infections, β-lactams are extensively used in human and veterinary medicine (23, 41). β-Lactam antibiotics can be divided into six different groups, the penicillins, cephalosporins, carbapenems, cephamycins, monobactams, and β-lactamase inhibitors. The predominant cause of resistance to β-lactam antibiotics in gram-negative bacteria is the production of β-lactamases, which are encoded chromosomally or on plasmids and inactivate β-lactams by hydrolyzing the four-membered β-lactam ring. On the basis of their primary structures, β-lactamases are divided into four classes (classes A, B, C, and D) (1, 9). Genes encoding the β-lactamases of classes A, B, and D are located on transferable plasmids or on the chromosome. Initially, class C β-lactamases were described as chromosomally encoded enzymes, but over the last 20 years, several plasmid-encoded transferable class C enzymes have been identified (35). In *E. coli*, resistance provided by class C β-lactamases can be plasmid encoded or due to the overexpression of the chromosomal *ampC* gene. The *E. coli* chromosomal AmpC enzyme differs from other class C chromosomal β-lactamases of the *Enterobacteriaceae* in that its production is not inducible but is constitutive and that AmpC production depends mostly on the strength of the *ampC* promoter. Many mutations in the *ampC* promoter of clinical strains have been described (12).

In gram-negative bacteria, resistance to extended-spectrum cephalosporins has been associated with the production of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated class C β-lactamases (4). Class A ESBLs (CTX-M or enzymes derived from TEM-1 and SHV-1) and plasmidic class C β-lactamases have already been described in *E. coli* but also in other bacteria including *Klebsiella*, *Salmonella*, *Proteus*, and *Enterobacter* species (2, 9, 35). ESBLs confer resistance to most β-lactam antibiotics, including oxyimino-β-lactams such as ceftazidime, ceftiofur, and aztreonam, but are not active against cephamycins, like cefoxitin, and carbapenems and they can be inactivated by clavulanic acid. This is in contrast to class C β-lactamases, which usually confer resistance to all β-lactams with the exception of dipolar ionic methoxy-imino-cephalosporins such as cefepime and the carbapenems (9, 32, 40).

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Since the first ESBL was detected in the mid-1980s, the number of different ESBLs has increased rapidly (http://www.lahey.org/studies/inc_webt.asp), but plasmidic class C β-lactamases have also taken their entry (9, 34, 35).

Antimicrobial resistance in commensal *Enterobacteriaceae* of food animals may play an important role in the ecology of resistance and may serve as an important reservoir for these transmissible resistance genes (31).

Recently, reports concerning *E. coli* and *Salmonella* strains carrying β-lactamases isolated from farm animals have been published worldwide (11, 22, 27, 46). Those reports focused on the characterization of β-lactamases and monitored the prevalence of broad-spectrum β-lactamases. Knowledge about the diversity of broad-spectrum β-lactamases among *Enterobacteriaceae* within a farm remains limited.

In Belgium, resistance to extended-spectrum cephalosporins has been reported in isolates of *Salmonella enterica* serovars Virchow and Infantis from poultry, food, and humans (6, 13). The diversity and prevalence of broad-spectrum β-lactamases among other *Enterobacteriaceae* and the epidemiology of these bacteria at the poultry farm level in Belgium are still unknown.

To better understand the epidemiology of ESBL- and class C β-lactamase-carrying bacteria in poultry, the prevalence of commensal ceftiofur-resistant *E. coli* in broilers was studied, the resistance phenotype of these isolates was determined, and their broad-spectrum β-lactamases were characterized.

MATERIALS AND METHODS

Sampling. A total of five broiler farms were visited. The farms were scattered randomly over Belgium. In two of the five farms, cattle were also present. One hundred or 89 cloacal samples per farm from healthy 5-week-old broilers were collected from as many chickens. Each cloacal sample was inoculated on ceftiofur (8 μg/ml)-supplemented MacConkey agar plates (Oxoid Ltd., Basingstoke, Hampshire, England). Plates were examined for growth after overnight aerobic incubation at 37°C. Colonies were further inoculated on Columbia agar with 5% sheep blood (blood agar; Oxoid) and phenotypically identified after overnight aerobic incubation at 37°C (33). One colony per plate was taken. Except for 17 samples, two colonies with different morphologies per plate were selected for further identification. Only *E. coli* isolates were retained for further testing.

Antimicrobial susceptibility testing. The antimicrobial susceptibilities of the *E. coli* isolates were determined using the Kirby Bauer disk diffusion test. Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (*E. coli* ATCC 25922). Briefly, after standardization of the inoculum in phosphate-buffered saline (0.5 McFarland standard) (Densimat; Biomérieux, Marcy l'Etoile, France), the inoculum was triple streaked (60° rotation of the round petri dishes within streaks) on Mueller-Hinton II agar (Oxoid), and antimicrobial tablets were then brought onto the medium by means of a dispenser (30). *E. coli* isolates were tested for resistance against β-lactams (ampicillin [33 μg], ceftazidime [30 μg], ceftriaxone [30 μg], cefepime [30 μg], ceftiofur [30 μg], aztreonam [30 μg], amoxicillin-clavulanic acid [30 and 15 μg, respectively], imipenem [15 μg], and cefoxitin [60 μg]) (Neo-Sensitabs; Rosco Diagnostica, Taastrup, Denmark) to confirm the presence of an ESBL or a class C β-lactamase. ESBL producers were identified when the marked synergistic effect between extended-spectrum cephalosporins and amoxicillin with clavulanic acid was observed. This β-lactam resistance phenotype is the ESBL phenotype. The absence of this synergistic effect but resistance to both cefoxitin and β-lactamase inhibitors (clavulanic acid) or reduced susceptibility to β-lactamase inhibitors and with reduced susceptibility to expanded-spectrum cephalosporins suggested the presence of a class C β-lactamase and is the AmpC phenotype.

Further susceptibility to aminoglycosides (apramycin [40 μg], gentamicin [40 μg], kanamycin [100 μg], neomycin [120 μg], and streptomycin [100 μg]), amphenicols (chloramphenicol [60 μg] and florphenicol [30 μg]), tetracycline (80 μg), quinolones (nalidixic acid [130 μg]), fluoroquinolones (enrofloxacin [10 μg]), trimethoprim (5.2 μg), and sulfonamides (240 μg) (Neo-Sensitabs; Rosco Diagnostica) was also assessed.

Intermediate zones of inhibition were counted as susceptible in this study.

rep-PCR. *E. coli* strains were genotyped using repetitive extragenic palindromic-PCR (rep-PCR) to assess the diversity of the isolates and to allow the selection of isolates for further analysis.

DNA was extracted by inoculating a single colony of each isolate from a blood agar plate into 1 ml of Luria broth. After overnight incubation at 37°C, cells were harvested after centrifugation at 16,000 × *g* for 5 min. The pellet was resuspended in 1 ml of distilled water, and cells were lysed by heating at 95°C for 5 min. Cellular debris was removed by centrifugation at 16,000 × *g* for 5 min.

rep-PCR was adapted from a method described previously by Rademaker and de Bruijn (36). The primers used were REP 1R (5′-III ICG ICG ICA TCI GGC-3′) and REP 2I (5′-ICG ICT TAT CIG GCC TAC-3′) (15, 16). PCR mixtures were prepared by using 2 μl template DNA, 12.5 μl PCR master mix {2.5 U/reaction *Taq* DNA polymerase, 1× PCR buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM Mg Cl_2] (pH 8.7)}, 200 μM deoxynucleoside triphosphate (Qiagen, Venlo, The Netherlands), and 1.5 μl of each primer (10 μM; Eurogentec, Seraing, Belgium) in a volume of 25 μl. PCR conditions were 95°C for 6 min; 30 cycles each of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; and a final step at 65°C for 16 min (15). Ten microliters of each mixture was separated on a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer at a constant voltage of 70 V for 18 h at 4°C. A 100-bp (100 to 3,000 bp) PCR molecular ruler (Bio-Rad, Nazareth, Belgium) was used as a standard. Gel images were analyzed with BioNumerics software (Applied Maths, Kortrijk, Belgium). Only PCR products that were 0.5 to 3.0 kb long were considered for analysis in this study. Dice similarity coefficients were determined in order to quantify the similarity between DNA fingerprints. A dendrogram was constructed by using the unweighted-pair group method with arithmetic averages.

Characterization of β-lactamases. For isoelectric focusing (IEF), cultures were grown overnight at 37°C in 10 ml of brain heart infusion broth with 100 μg/ml ampicillin. Bacterial suspensions were disrupted by sonication (two bursts of 30 s on ice at an amplitude of 12 μm in an MSE Ultrasonic Disintegrator Mark II) and centrifuged (15 min at 10,000 × *g* at 4°C). The supernatants containing the crude enzyme extract was loaded onto a Ampholine PAGplate pH 3.5 to 9.5 IEF gel (GE-Amersham Bioscience, Uppsala, Sweden) and run at 1,500 V for 90 min. β-Lactamase bands were visualized by overlaying the gel with 0.1 mg/ml nitrocefin (Oxoid). The pI values were determined and compared with the pI values of known β-lactamases: PER-1 (pI 5.3), TEM-24 (pI 6.5), VEB-1 (pI 7.4), and CMY-4 (pI 9.2).

Based on the IEF results, PCR for the detection of genes encoding TEM-, SHV-, CTX-M-, and CMY-type enzymes was performed on genomic DNA extracted as described above. PCR mixtures were prepared using 20 μl PCR master mix {2.5 U/reaction *Taq* DNA polymerase, 1× PCR buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM Mg Cl_2] (pH 8.7), 200 μM deoxynucleoside triphosphate (Qiagen)}, 2.4 μl of each primer (10 μM; Eurogentec), and 2 μl template DNA in a volume of 40 μl. Primers specific for these β-lactamases and the conditions for these reactions were reported previously (19, 34). Isolates that contained a $bla_{CTX\text{-}M}$-type gene were further analyzed using primers to differentiate between CTX-M-1, CTX-M-2, and CTX-M-9 groups (19). Isolates that tested positive for the bla_{CMY} gene were further analyzed to differentiate between the $bla_{CMY\text{-}1}$ and $bla_{CMY\text{-}2}$ genes (34). Positive controls (*Enterobacter aerogenes* 3/30187 [$bla_{TEM\text{-}24}$], *E. aerogenes* 3/30186 [$bla_{SHV\text{-}4}$] [obtained from G. Claeys], *Klebsiella pneumoniae* 2974 [$bla_{CMY\text{-}4}$] [obtained from G. Arlet], and *S. enterica* serovar Virchow 968/03 [$bla_{CTX\text{-}M\text{-}9}$] [obtained from S. Pournaras]) as well as negative controls were included in each type of PCR. All PCR products were purified using a Nucleospin Extract II kit (Macherey-Nagel GmbH & Co. KG) and sequenced using an Applied Biosystems GeneAmp PCR 9700 sequencer. The PCR primers were used for sequencing. The obtained nucleotide sequences were compared with those previously described for *bla* genes (BLAST database [http://www.ncbi.nlm.nih.gov/BLAST/]).

The chromosomal *ampC* gene including its promoter and attenuator regions was amplified and sequenced by using primers Int-B2 (5′-TTCCTGATGATCG TTCTGCC-3′) and Int-HN (5′-AAAAGCGGAGAAAAGGTCCG-3′) as described previously (29). PCR amplifications yielded a 1,315-bp amplification product. Mutations in the promoter and attenuator regions were studied by comparing the sequences with the sequence of the same region in the *E. coli* K-12 *ampC* gene (24).

RESULTS

Bacterial isolates and antimicrobial susceptibility. Two hundred ninety-five ceftiofur-resistant *E. coli* isolates were ob-

TABLE 1. Distribution of β-lactam resistance phenotypes among ceftiofur-resistant cloacal *E. coli* isolates from broilers on each farm

Farm	No. of cloacal samples collected	No. of ceftiofur-resistant *E. coli* isolates	No. (%) of ESBL producers	No. (%) of AmpC producers	No. (%) of combinations of ESBL and AmpC
1	89	55	16 (29)	39 (71)	0 (0)
2	100	27	15 (55)	12 (45)	0 (0)
3	100	70	0 (0)	70 (100)	0 (0)
4	100	75	55 (73)	5 (7)	15 (20)
5	100	68	47 (69)	1 (1)	20 (30)
Total	489	295	133 (45)	127 (43)	35 (12)

tained from the 489 samples. The distribution of β-lactam resistance phenotypes among these isolates on the different farms is shown in Table 1. We identified 133 (45%) *E. coli* isolates as being ESBL producers and 127 (43%) *E. coli* isolates as being AmpC (plasmidic or chromosomally) β-lactamase producers. A third β-lactam resistance phenotype exhibited the combination of an ESBL phenotype with an AmpC β-lactamase phenotype. This suggested the presence of both an ESBL and a class C β-lactamase in one isolate. This phenotype was detected in 35 (12%) *E. coli* isolates and only in farms 4 and 5. ESBL and AmpC producers were observed in most farms with the exception of farm 3. In this farm, only the AmpC phenotype was seen.

A summary of results of disk diffusion tests is shown in Table 2. All the *E. coli* isolates showed resistance to ceftiofur. No resistance to imipenem, apramycin, and florphenicol was observed. Resistance to chloramphenicol, gentamicin, kanamycin, neomycin, and enrofloxacin was below 10%. In contrast, 64% of the isolates were resistant to trimethoprim, 66% were resistant to sulfonamides, and nearly half were resistant to nalidixic acid and tetracycline. Major differences in resistance percentages were seen between the different farms (Table 2). Resistance to non-β-lactams in farm 1 was very low. Only resistance to nalidixic acid, tetracycline, and trimethoprim was observed. Farms 2, 4, and 5 showed resistance to sulfonamides and trimethoprim of nearly 100%. Of the 295 *E. coli* isolates, 225 isolates were multidrug resistant, showing resistance to two or more non-β-lactam antimicrobial agents. Nineteen percent of the isolates showed resistance to β-lactams only, 5% of the isolates were resistant to one additional antimicrobial agent, and the other 76% were resistant to at least two or more antimicrobials. Some strains (4%) were resistant to up to eight additional antibiotics.

Genetic background of *E. coli*. Fifty-one different Rep profiles were identified, and each profile was regarded as a single clone. There were no common clones between farms. One isolate per clone was selected for further β-lactamase analysis.

β-Lactamase characterization. Of the 51 selected cephalosporin-resistant *E. coli* isolates, crude protein extracts were characterized by IEF. Different pIs were observed (5.4, 6.0, and 8.0 to 9.0) among these 51 isolates.

Based on these results, PCRs with primers specific for the bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$-type, and bla_{CMY} genes were performed. Twenty-six (55%) isolates were found to carry a bla_{TEM} gene, 22 (43%) isolates carried the $bla_{\mathrm{CTX\text{-}M}}$-type gene, and 25 (49%) isolates carried the bla_{CMY} gene. No bla_{SHV} genes were found. Sequence analysis revealed the following ESBLs: TEM-52 ($n = 7$), TEM-106 ($n = 1$), CTX-M-1 ($n = 14$), CTX-M-2 ($n = 4$), CTX-M-14 ($n = 3$), and CTX-M-15 ($n = 1$) (Table 3). The only plasmidic AmpC β-lactamase found in this study was the CMY-2 enzyme ($n = 25$). Mutations in the promoter and attenuator regions of the chromo-

TABLE 2. Resistances to β-lactam and non-β-lactam antimicrobials of ceftiofur-resistant β-lactamase-producing *E. coli* isolates from 5-week-old broilers on five different farms

Antimicrobial agent	No. (%) of resistant strains					Total no. of isolates (%)
	Farm 1 ($n = 55$)	Farm 2 ($n = 27$)	Farm 3 ($n = 70$)	Farm 4 ($n = 75$)	Farm 5 ($n = 68$)	
Amoxicillin-clavulanic acid	1 (1.8)	12 (44)	70 (100)	20 (27)	20 (30)	123 (41.7)
Ceftazidime	51 (93)	27 (100)	70 (100)	75 (100)	61 (90)	284 (96.3)
Ceftiofur	55 (100)	27 (100)	70 (100)	75 (100)	68 (100)	295 (100)
Cefepime	17 (31)	18 (67)	0 (0)	71 (95)	67 (99)	188 (63.7)
Ceftriaxone	17 (31)	27 (100)	35 (50)	72 (96)	67 (99)	218 (73.9)
Aztreonam	26 (47)	17 (63)	2 (3)	58 (77)	48 (71)	151 (51.2)
Cefoxitin	33 (60)	12 (44)	70 (100)	20 (27)	20 (29)	155 (52.5)
Kanamycin	0 (0)	15 (56)	2 (3)	5 (7)	0 (0)	22 (7.5)
Gentamicin	0 (0)	0 (0)	0 (0)	0 (0)	12 (17)	12 (4)
Streptomycin	0 (0)	24 (89)	21 (30)	11 (15)	33 (49)	89 (30.2)
Neomycin	0 (0)	14 (52)	1 (1)	2 (3)	1 (1)	18 (6.1)
Chloramphenicol	0 (0)	11 (41)	2 (3)	0 (0)	1 (1)	14 (4.7)
Tetracycline	19 (35)	25 (93)	23 (33)	47 (63)	28 (41)	142 (48.1)
Nalidixic acid	42 (76)	14 (52)	28 (40)	7 (9)	54 (79)	145 (49.5)
Enrofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	25 (37)	25 (8.7)
Trimethoprim	13 (24)	23 (85)	18 (26)	69 (92)	67 (99)	190 (64.4)
Sulfonamides	0 (0)	27 (100)	29 (41)	72 (96)	66 (97)	194 (65.8)

TABLE 3. Distribution of ESBL and plasmidic class C β-lactamases among 51 ceftiofur-resistant cloacal *E. coli* isolates obtained on five different broiler farms

Enzyme	β-Lactamase	No. (%) of isolates					Total no. of isolates (%)
		Farm 1 ($n = 6$)	Farm 2 ($n = 7$)	Farm 3 ($n = 10$)	Farm 4 ($n = 15$)	Farm 5 ($n = 13$)	
Narrow-spectrum β-lactamase	TEM-1	2 (33)	2 (29)	5 (50)	7 (47)	5 (38)	21 (41.2)
ESBL	TEM-52	1 (17)	3 (43)	0 (0)	2 (13)	1 (8)	7 (13.7)
	TEM-106	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	1 (2)
	CTX-M-1	0 (0)	3 (43)	0 (0)	2 (13)	5 (38)	10 (19.6)
	CTX-M-2	0 (0)	0 (0)	0 (0)	4 (27)	0 (0)	4 (7.8)
	CTX-M-14	0 (0)	0 (0)	0 (0)	0 (0)	3 (23)	3 (5.9)
	CTX-M-15	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	1 (2)
Plasmidic class C β-lactamase	CMY-2	5 (83)	1 (14)	10 (100)	4 (27)	1 (8)	21 (41.2)
ESBL + plasmidic class C β-lactamase	CTX-M-1+ CMY-2	0 (0)	0 (0)	0 (0)	1 (7)	3 (23)	4 (7.8)

somal *ampC* gene were found in eight isolates and in association with $bla_{\text{CMY-2}}$ ($n = 4$) genes or ESBL ($bla_{\text{CTX-M-1}}$ [$n = 3$] and $bla_{\text{TEM-52}}$ [$n = 1$]) genes as shown in Table 4. Important promoter mutations in the −35 box and −10 box and at positions −42 and −11 were not detected. The distance between the two conserved regions also plays an important role in the strength of the promoter, and the optimal distance is 17 bp (12). In our isolates, the distance was 16 bp.

In addition, TEM-1 was found in 21 isolates. This enzyme was found in combination with all β-lactamases detected, except TEM-52 and TEM-106.

At the farm level, many differences between farms were noted (Table 3). The CMY-2 gene was found in all farms and was the single gene causing extended resistance to β-lactam antibiotics in farm 3. The other farms were characterized by different TEM, CTX-M, and CMY enzymes as shown in Table 3. The ESBLs TEM-52 and CTX-M-1 were present in multiple farms, in contrast to the other ESBLs. The CTX-M family was the most abundant family of ESBLs. In three isolates, the combination of an ESBL (CTX-M-1) with a plasmidic AmpC β-lactamase (CMY-2) was found. This phenomenon was seen in farm 4 and farm 5.

TABLE 4. Mutations in the *ampC* promoter/attenuator region in CMY-2- and ESBL-producing strains

Farm	Isolate	*bla* gene(s) detected	Mutation(s) in *ampC* promoter/attenuator region
1	4	$bla_{\text{CMY-2}}$	−28 (G→A) and +17 (C→T)
1	47	$bla_{\text{CMY-2}}$	−18 (G→A), −1 (C→T), and +58 (C→T)
2	16	$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-1}}$	−18 (G→A), −1 (C→T), and +58 (C→T)
2	34	$bla_{\text{TEM-52}}$	−28 (G→A)
2	52	$bla_{\text{CTX-M-1}}$	−18 (G→A), −1 (C→T), and +58 (C→T)
3	12	$bla_{\text{CMY-2}}$	+58 (C→T)
3	66	$bla_{\text{TEM-1}}$ and $bla_{\text{CMY-2}}$	−18 (G→A), −1 (C→T), and +58 (C→T)
4	51	$bla_{\text{CTX-M-1}}$	−18 (G→A), −1 (C→T), and +58 (C→T)

DISCUSSION

In this study, we demonstrated that ceftiofur-resistant *E. coli* strains are frequently present in cloacal samples from broilers. This is remarkable since cephalosporins are not allowed for use in poultry in Belgium. Although ceftiofur was approved in April 1990 for the parenteral treatment of 1-day-old chickens to prevent *E. coli* septicemia, this product was already withdrawn from the Belgian market in January 2000. Ceftiofur and other cephalosporins with an extended spectrum can be used in pigs, cattle, and pet animals.

Approximately one-half of the ceftiofur-resistant strains were also resistant to tetracycline and nalidixic acid, and more than half were resistant to trimethoprim and sulfonamides. Otherwise, the prevalence of resistance against antimicrobials other than the β-lactams was rather low. For *Enterobacteriaceae*, resistance to quinolones is generally acquired in a two-step mutation process. A first mutation in the quinolone resistance-determining region of the *gyrA* gene gives full resistance to narrow-spectrum quinolones, such as nalidixic acid, and decreased susceptibility to fluoroquinolones. A second mutation in one of the *gyr* or *par* genes gives full resistance to fluoroquinolones (39). We found nearly 50% of the strains to be resistant to nalidixic acid, and fluoroquinolone resistance was detected in only one farm. The high prevalence of nalidixic acid resistance, however, increases the risk for the development of fluoroquinolone resistance.

Many of the ceftiofur-resistant *E. coli* isolates also showed acquired resistance to several other classes of antimicrobials, and some were highly multiresistant. At this time, resistance to therapeutically important antimicrobial agents is becoming a particular concern. The genetic homology among all isolates was relatively low, even within a farm, as expected, since *E. coli* has a polyclonal population structure.

Although only five farms were sampled, a multitude of six different ESBLs and one plasmidic AmpC β-lactamase were detected, indicating a high diversity of resistance genes in *E. coli* strains in Belgian broilers.

The CTX-M group of β-lactamases was the predominant ESBL type identified in our isolates, and the CTX-M-1 enzyme

was the most abundant. It must be noted that isolates carrying a CTX-M enzyme were also resistant to sulfonamides and trimethoprim. The CTX-M-2 enzyme found in *Salmonella* from Belgian poultry has been localized in a complex class 1 integron, which also contains the resistance genes of sulfonamides and trimethoprim (6, 21). Previously reported analyses of the surrounding regions of CTX-M enzymes have shown the association of β-lactamase genes with the insertion sequence IS*Ecp1* (19). Furthermore, those analyses confirmed the predominant role of IS*Ecp1* in the mobilization of $bla_{\text{CTX-M}}$ genes of the CTX-M-1 group (of human isolates) and the presence of a novel complex class 1 integron (18). The CTX-M-14 enzyme of a clinical *E. coli* isolate and the CTX-M-9 enzyme of a *Salmonella* isolate from healthy food animals were also part of a complex class 1 integron (3, 37). Further analysis to determine the genetic environment of these genes is needed to confirm whether the CTX-M enzymes found in our study are also located in similar structures. Also, in Belgium, CTX-M-2 was reported in extended-spectrum cephalosporin-resistant *S. enterica* serovar Virchow strains from poultry and humans (6), and CTX-M enzymes belonging to groups 1, 2, and 9 emerged in human *E. coli* strains in 2002 (38). The presence of CTX-M-type extended-spectrum β-lactamases in farm animals has also been reported in other countries (7, 17, 28).

Another class A ESBL reported among several farms was the TEM-52 enzyme. In Belgium and France, the presence of TEM-52 was demonstrated in *Salmonella enterica* serovars isolated from poultry and humans (13). In The Netherlands, TEM-52 was the most common ESBL detected in *Salmonella* and was found mainly in *Salmonella* serovar Paratyphi B from poultry, poultry products, and humans (22). Other European reports also described the presence of TEM-52 *E. coli* producers in animals (7, 14).

Another TEM-type ESBL found in one isolate was the TEM-106 enzyme. This TEM derivative differs from the TEM-52 enzyme in only four nucleotides. To our knowledge, this is the first time that the presence of this enzyme has been described in bacteria from food-producing animals.

Of the plasmidic class C β-lactamases, only the CMY-2 enzyme was identified. CMY-2 was the most frequently isolated β-lactamase among farms and was present in all farms investigated in this study.

E. coli and *Salmonella* strains carrying the CMY-2 β-lactamase have already been reported in food-producing animals and humans in Europe, Africa, Asia, and the United States (5, 7, 20, 26, 28, 45, 46). In the United States, CMY-2 is highly prevalent among *E. coli* and *Salmonella* strains from food-producing animals and humans. The transfer of CMY-2 between different bacterial species and between animals and humans has been suggested (46). This is the first description of the CMY-2 enzyme in *E. coli* from animals in Belgium.

The mutations in the promoter and attenuator regions of the *ampC* gene were probably not important for the resistance phenotypes found. Mutations at positions −42, −32, and −11 were not found in this study, although these mutations would be important for the overexpression of the chromosomal *ampC* gene (12). In the ESBL strains, no additional resistance to amoxicillin with clavulanic acid or the lack of inhibition effect of clavulanic acid was seen. However, it has been suggested that the mutation at position −18, creating a new −10 box and also found in our study, plays an important role in the expression of the chromosomal *ampC* gene (12, 42). Mutations at positions −28, −18, −1, and +58 in combination with an ESBL in clinical *E. coli* isolates were described previously (10).

In the strains expressing a plasmid-mediated *ampC* gene, resistance to cefoxitin might be explained by the expression of the $bla_{\text{CMY-2}}$ gene, although the effect of the chromosomal *ampC* gene on the resistance of mutations in the promoter (positions −18 and −28) and the attenuator (position +17) region found in our study cannot be evaluated. The effect could have been masked. Not only mutations in the promoter region but also mutations in the attenuator region (position +17) are important and are thought to contribute to AmpC overproduction by destabilizing the hairpin structure, resulting in increased transcription (42). Mutations at positions −18, −1, and +58 in association with a $bla_{\text{CMY-2}}$ gene in farm animals were described previously (27). Further studies are necessary to determine the real role of the mutations found in our study.

A possible explanation for the high prevalence of β-lactamases with an extended spectrum among *E. coli* strains from broilers may be coselection due to the use of other non-β-lactam antimicrobials. Resistance genes against these antimicrobial agents may be located in the same mobile genetic element as genes encoding broad-spectrum β-lactamases (e.g., transposons or integrons) (8, 21). Further studies are needed to confirm this hypothesis.

The presence of ESBLs and *ampC* genes in the microbiota of broilers may pose a human health hazard since these bacteria may represent a reservoir of resistance genes for pathogens causing disease in humans and animals (43, 44). Further studies on the location and transfer possibilities of these genes should be carried out to elucidate if a common reservoir exists.

In conclusion, this is the first detailed documentation of a high diversity of β-lactamases with an extended spectrum in *E. coli* at the poultry farm level. This finding necessitates a follow-up evaluation of extended-spectrum β-lactam resistance in commensal *E. coli* of poultry in order to be able to estimate the public and animal health burden.

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